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Detection and Genotyping of the Hepatitis C RNA in Tear Fluid From Patients With Chronic Hepatitis C

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Tear fluid from 51 patients with chronic hepatitis C virus (HCV) infection was analyzed for the presence of the hepatitis C RNA to assess the potential role of this fluid in virus transmission. HCV sequences were amplified from sera and tear fluids by nested polymerase chain reaction using primers from the 5′ non coding region of the virus genome. Positive samples were genotyped by the LiPA™ procedure. HCV RNA was detected in 76.5% (39/51) of the sera and in 9.8% (5/51) of the tear fluid samples. The presence of the RNA in the tear fluid was independent of the severity of the hepatitis and of the viral load as measured by the branched DNA™ assay. The genotypes of the tears and serum isolates were different for two patients. For another patient, the HCV RNA was positive in the tear sample but negative in the serum sample. These findings suggest that tear fluid may transmit HCV but the source of HCV RNA in this fluid needs to be better understood. ***J. Med. Virol. 51: 231–233, 1997.***

KEY WORDS: hepatitis C RNA; tear fluid; HCV genotypes

INTRODUCTION

The hepatitis C virus (HCV) is mainly transmitted by the parenteral route. However there is no identifiable source of infection in approximately 50% of patients with HCV infection. The virus may be transmitted via body fluids [Fried et al., 1992; Liou et al., 1992; Mariette et al., 1995; Young et al., 1993], but few studies show the presence of HCV RNA in tear fluid [Feucht et al., 1994, 1995; Salinas et al., 1996; Shimazaki et al., 1994]. To study further whether tear fluid is involved in HCV transmission, we screened the serum and the tear fluid from patients with chronic hepatitis C virus for the presence of viral genome by the polymerase chain reaction. (We first defined chronic hepatitis as HCV antibodies by RIBA™ and a positive liver biopsy.)

MATERIALS AND METHODS

Fifty-one patients with known hepatitis C chronic infection were investigated; their serum alanine aminotransferase (ALT) levels were from normal to 12-fold the normal upper limit. Ten healthy subjects negative for serum anti-HCV antibodies served as controls. Serum and tear fluid were collected on the same day; the tear fluid was drawn off with a sterile microcapillary tube and transferred immediately to a microtube containing guanidium isothiocyanate and was stored in liquid nitrogen until analysis.

RNA was extracted from 50 μl of both serum and tear fluid by the same technique using RNazol (Bioprobe Systems, Montreuil Sousbois, France), chloroform and isopropranol precipitation. cDNA was synthetised in the presence of 1μM of the primer KB2 (Genset, Paris, France) located in the 5′ non coding region of the viral genome (5′-CAC TCG CAA GCA CCC TAT CA-3′) in a mixture containing 20 U RNAsin (Boehringer, Mannheim GmbH, Mannheim, Germany), 200 U RT-MLV (Gibco, Life Technologies, Gaithersburg, MD), 10 mM of each dNTP (Perkin Elmer Cetus, Norwalk, CT), 200 mM DTT (Gibco) and RT buffer (Gibco) for 1 hr at 37°C. The cDNA was amplified with *Taq* polymerase (Perkin-Elmer Cetus) in a thermal cycler (DNA thermal cycler, Perkin Elmer Cetus) in the presence of the biotinylated primers KB2 and KB3 (5′-CTG TGA GGA ACT ACT GTC TT-3′) (Genset); the PCR consisted of 3 min at 94°C then 40 cycles of 1 min each of 94°C, 50°C and 72°C and final incubation at 72°C for 10 min. A second round of amplification was done with a different set of "nested"

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TABLE I. Clinical and Biological Features of the Five Patients With HCV RNA in Tear Fluid

Patient No.	Age (year)	Sex	ALT	Histology (Knodell's score)	HCV genotype blood/tear	Treatment
1	52	F	N	N	1b/1b	No
2	60	M	2N	CPH (4)	la+1b/1a+1b	No
3	56	F	3N	CPH (3)	1a/1b	Yes
4	54	F	2N	CPH (5)	3a/1b	Yes
5	63	F	2N	Cirrhosis	Negative/1b	Yes

N, normal.
CPH, chronic persistent hepatitis.
Treatment*: interferon α withdrawn 6 months previously.

primers (Innogenetics, NV, Ghent, Belgium) [Stuyver et al., 1993] corresponding to the highly conserved 5′ non coding region of the HCV genome. Three control groups of dense, average and weak concentrations of HCV RNA were used. The final amplified products were visualized under UV light after separation by a 2% agarose gel electrophoresis. The HCV-RNA positive samples were genotyped using the InnoLiPA™ assay (Innogenetics) according to the manufacturer's instructions. Serum HCV RNA was quantified using the branched DNA™ assay (Quantiplex™ HCV 1.0 Chiron Corporation, Emeryville, CA) keeping in mind that this assay version does not detect the different HCV genotypes with the same efficiency.

In parallel, two different controls were carried out. First, the β globin gene was amplified to assess the absence of any cellular contamination in each of the tear fluid. Second, in order to verify that the presence of polysaccharides in tear fluid was not responsible for PCR inhibition, we added 50 μl of serum known to contain HCV RNA to a tear sample from a healthy subject.

RESULTS

HCV RNA was detected by PCR in the sera of 76.5% (39/51) of the patients with chronic HCV infection. The genotype distribution according to the Simmonds' classification [Simmonds et al., 1994] was as follows: 1b (n = 18); 1a (n = 6); 1 (n = 1); 2a (n = 2); 3a (n = 7); 4a (n = 3); 1a + 1b (n = 1); undetermined (n = 1).

The viral genome was detected in the tear sample from four of the 39 patients with serum positive for HCV RNA and from one of the 12 patients with serum negative for HCV RNA. Table I shows the characteristics of the patients with HCV RNA in tear fluid. Genotype of the virus in the tear sample was the same as found in the blood in two patients (numbers 1 and 2), but was surprisingly different in the two others (numbers 3 and 4). These results were confirmed by genotype determination by direct sequencing of the amplified fragments. Moreover, one patient (number 5) was found to be genotype 1b positive in tear fluid but HCV RNA negative in the blood.

Sera and tears from all 10 controls were negative for HCV RNA. The absence of cellular contamination of the tear fluid was demonstrated by failure to amplify the β globin gene. The addition of HCV RNA-positive serum in a tear sample scored positive, indicating that the tear fluid did not contain any PCR inhibitors.

Among the 39 sera scored RNA positive by the PCR technique, HCV RNA was detectable in only nine sera by the branched DNA™ assay. The titers were between 10.3×10^5 Eq/ml and 150×10^5 Eq/ml after using a correction factor to obtain accurate values according to different genotypes. None of these sera were from the patients with HCV RNA-positive tear fluid.

DISCUSSION

We tested for the HCV genome in the tear fluid from patients with chronic hepatitis C. HCV RNA was found in around 10% of the tear samples contrasting with previous reports [Feucht et al., 1994, 1995; Shimazaki et al., 1994] that found HCV RNA in 85% to 100% of the tear fluid. The reasons for these divergent findings are unclear. The PCR technique is extremely sensitive but it is also prone to false positive results because of contamination with extraneous HCV RNA. It is noteworthy that in these previous studies, the tear fluid was collected with Schirmer strips or after instillation of saline solution that may result in contamination with blood or cells. Our inability to detect HCV RNA in numerous tear fluid samples may be due to the low viral load in serum. But few of our patients had high levels of serum HCV RNA as compared to the different controls after the first round of PCR (data not shown) and as assessed by the level of serum equivalents with the branched DNA™ assay.

The five patients with HCV RNA in tears had mild hepatitis C infection according to the ALT levels and to the histological damage (Table I). Furthermore, in these patients, the serum HCV RNA was detected only after the second round of PCR (nested PCR). This is consistent with the negative bDNA™ assay. The presence of the RNA in tear fluid was independent of the severity of the hepatitis. Therefore, the serum HCV RNA level is not predictive of the presence of the HCV RNA in the tear fluid. This contrasts with other body fluids as recently shown by Caldwell et al. [1996].

Genotype 1b was found to be the most frequent genotype which is consistent with a study done in France in older patients [Pawlotsky et al., 1995] (the mean age of our cohort was ≈45 years), with the exception of HIV-positive patients [Mendel et al., 1995]. The sequences of the amplified fragments confirmed the genotypes indicated by the LiPA™ procedure. It is noteworthy that genotype 1b is detected in all the tear fluid samples. Moreover, we found point mutations which allowed us

to confirm the presence of different viral strains which rules out the possibility of contamination with a 1b sequence. Sequence of these samples in the NS5 variable region of the viral genome is now being investigated in order to further confirm the absence of outside contamination. For patient number 2, the association of genotypes 1a and 1b was confirmed with a relative proportion of 1b being higher in blood. Unexpectedly, the genotype of the isolates from blood was not the same as that from the tear fluid for patients numbers 3 and 4; moreover, patient number 4 had genotype 1b in tear fluid identical to that found in his serum the previous year. Patient number 5 had HCV RNA in tear fluid contrasting with serum negative for HCV RNA (Table I). These three patients (numbers 3, 4, 5) with divergent findings had previously been treated with interferon α but they relapsed after interferon withdrawal. Possibly the treatment had selected a new strain of virus found in the circulation whereas the isolate in the tear fluid is a remnant of the initial largely eliminated strain. Alternatively, these inconsistent cases may simply correspond to a mixed infection that could not be detected in both sites.

HCV RNA was detected in the tear fluid infrequently. This fluid therefore is potentially responsible for nonblood transmission of hepatitis C virus. Further investigation is needed to determine the source of the HCV RNA in the tear fluid: transsudation or active replication? Appropriate disinfection of equipment in ophthalmological practice would appear to be necessary in view of the presence of HCV in ocular fluid.

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